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Studies of the Effects of Some of the Constituents of Tobacco Smoke on Various Strains of Tissue Cells Cultivated In Vitro.

Rather extensive studies of the effects of two tobacco smoke constituents are nearing completion. Acetaldehyde and acetone were selected primarily because of their relatively high quantity, in comparison to others, in the gaseous phase of tobacco smoke. According to analyses, both of these two substances occur in the quantity of two milligrams per smoke of one cigarette.

Both short term experiments involving exposure times of less than one month and long term experiments, the so called time-concentration experiments, involving approximately six months of exposure have been conducted.

Four cell strains have been used in investigating these substances. They are Earle's strain L cells, Gey's HeLa cells, Mouse liver epithelial cells and Human skin cells. Stock cultures of these cells were maintained in T-60 flasks, at a constant temperature of 37.5°C in Earle's NCTC #109 medium supplemented with 10% horse serum. The medium was changed three times a week. Subculturing of the Liver cells and L cells was done routinely every week. In the cases of Skin cells and HeLa cells subculturing was frequently done as often as every three or four days. New and young T-60 cultures were gassed with a filtered 5% carbon dioxide and air mixture. The gassing procedure was used to assist in maintaining proper pH of the medium since it utilizes a carbonate buffer.

Checks on the toxicity and possible influence of these substances upon rate of proliferation were made periodically by the replicate method of culture. The replicate cultures were maintained at a constant temperature of 37.5°C, and received changes of appropriate medium three times a week. The experimental cells were usually exposed to the medium containing either acetaldehyde or acetone 48 hours after replication. Gassing of the Carrel cultures was done at every medium change throughout the experiments.

Two or 3 times a week 3 Carrel flasks from each group of control and experimental cultures were selected at random and sacrificed for enumeration. The medium of the cultures selected for counts was discarded and a known quantity of 0.1M solution of citric acid was added to each culture. They were placed in the incubator at 37.5°C for 2 to 3 hours. Nuclei counts were made by means of the Coulter Electronic Counter.

Data were accumulated in the form of recorded microscopical observations of the general appearance of the cells, photomicrographs of the cultures, and population growth curves.

Future plans call for investigating the effects of other individual constituents of tobacco smoke, whole tobacco smoke and some of the tar fractions on cell cultures in vitro. In some instances, when deemed advisable, time-concentration experiments will be carried out. Tentatively it is planned that some of the cell cultures from the time-concentration experiments will be used in injection experiments, involving mice, to determine if possible the acquisition of tumorgenic capacities by the cells.

A summary of the investigations of the short term experiments and the first 2 series of the acetaldehyde time-concentration experiments which were conducted, is presented here. Results of the acetone short term experiments and the final series of the acetaldehyde time-concentration experiments will be included in the next progress report.

ACETALDEHYDE

Short term experiments

All four cells types previously mentioned have been used in these investigations. Six different concentrations of acetaldehyde were used, two concentrations in each of three series: 8.0 mg and 2.0 mg per ml; 1.0 mg and 0.01 mg per ml; and 0.5 mg and 0.05 mg per ml. To prepare the experimental medium, the following procedure was used. The acetaldehyde, flasks, pipettes and regular medium were chilled. This was considered advisable because of the extremely low boiling point, 20°C, of acetaldehyde. Chilled acetaldehyde was quickly added to the cold medium and mixed thoroughly. Dilutions were made immediately. The cold medium was filtered, bottled in sterile 125 ml and 50 ml boiling flasks, tightly stoppered and stored under refrigeration. The medium was filtered this one time in order to minimize loss of acetaldehyde by vaporization.

Cells from 7 to 10 day old replicate cultures in Earle's NCTC #109 medium plus 10% horse serum were set up in Carrel D-3.5 flasks. After the cultures became well established, usually after 48 hours, they were exposed to the appropriate concentrations of acetaldehyde. Controls were maintained. Cell counts were made daily the first 3 and 4 days and from then on usually every other day. The usual techniques and precautions for handling such cultures were followed.

No conclusions were drawn from the short term experiments except in the cases where the concentrations were obviously toxic. The main objective of the short term experiments was to determine concentrations to be used in the long term or time-concentration experiments. The effects of the short term exposure are described in respect to morphological changes and populations.

Series 1. One experiment with concentrations of 8.0 mg and 2.0 mg acetaldehyde per ml of medium was made with each of L cells, Liver cells, and Skin cells. Morphological changes were soon visible in cells exposed to 8.0 mg per ml. The most conspicuous changes were the rounding up of the cells and subsequent detachment.

In the case of the cultures receiving 2.0 mg per ml, fewer cells were rounded and detached. In fact, to all outward appearances these cells continued to resemble the controls. Only upon observations of the resistance of cells to the citric acid preparatory to counting, rapid disintegration of the cells when returned to normal medium, and the use of time-lapse photography did not become apparent that these cells were dead and probably fixed.

Eight and 2 mg acetaldehyde per ml proved toxic to these cells in a matter of hours.

Series 2. Two experiments with concentrations of 1.0 and 0.01 mg of acetaldehyde per ml of medium were made with each in the case of Skin cells, HeLa cells and Liver cells, only one test was made with L cells.

The effects of 1.0 mg per ml were soon evident in that the cells rounded and detached; in the case of Liver cells within 1½ to 2 hours; in HeLa cells within 3½ hours.

In respect to 0.01 mg per ml, no definite morphological alterations were recognized under ordinary microscopical examination. There were times, after a week or more of exposure, when it did appear as though the experimental cells were not as healthy as the controls. The population growth curves constructed from the cell counts suggested a slight inhibitory effect. Since only two tests were made and the time of exposure was relatively short, no conclusions were drawn. It was felt that the indication of an effect was sufficient to use this concentration in the time-concentration experiments. Series 3. One test with the concentrations of 0.5 and 0.05 mg acetaldehyde per ml of medium was made with each of the four cell strains.

Cells treated with the 0.5 mg per ml concentration began to round and detach within a few hours. As in the case of cells treated with 0.1 mg per ml detachment became extensive enough that the cultures were discarded within three to four days. It was interesting to note that cells treated with the 0.5 mg per ml, although rounded and detached, were not killed immediately. A single check on the percentage of survival was made on HeLa cells, Liver cells and L cells using erythrosin B stain. Erythrosin B stains dead cells quickly and by using the hemocytometer, counts were made by which percent of survival was determined. In the case of L cells, 48 to 96 hours exposure resulted in killing all cells; with HeLa cells, 50% were surviving at the end of 46 hours; all of the Liver cells succumbed between 25 hours and 48 hours exposure, the greatest rate of death being between 4 and 25 hours of exposure.

The results obtained with the cells exposed to 0.05 mg per ml were similar to those obtained with the concentration of 0.01 mg per ml. Morphological changes were not detected by the usual microscopical examinations. Population curves again suggested a slight inhibitory effect. The indication was felt sufficient to use this concentration also in the time-concentration studies.

Time-Concentration Experiments

In these experiments three cell strains were employed, Mouse Liver cells, Earle's strain L cells, and HeLa cells. The two concentrations of acetaldehyde used were 0.01 and 0.05 mg per ml of medium.

Nine replicate cultures were set up in T-60 flasks by pooling 3 stock cultures of Liver cells. At the time of the second medium change (5 days later) the nine flasks were randomly divided into three groups. One of the groups was maintained on Earle's NCTC #109 medium plus 10% horse serum and carried as the control group. Another received the same medium plus 0.01 mg per ml of acetaldehyde. The third group was exposed to the same medium as group one plus 0.05 mg per ml of acetaldehyde. The same procedure was followed for L cells and HeLa cells.

All 27 cultures constituted the parent stock cultures of control and experimental cells for the entire experiment. They were kept at a constant temperature of 37.5°C. The medium was changed every 48 hours with the exception of the week-ends when the interval of time between changes was 72 hours. Every week the Liver cells and L cells were subcultured, while HeLa cells were often subcultured more frequently. Periodically, replicate cultures were begun in Carrel flasks with cells obtained from the controls, the 0.01 mg and the 0.05 mg cultures. Cell counts were made from time to time so that proliferation rates could be ascertained.

Series 1.

Mouse Liver cells:

Twenty-three days after the parent stock cultures had been established, replicate cultures were made in Carrel flasks with cells from the control and each of the two experimental groups. The initial inoculum for each set of Carrel flasks was slightly more than 300,000 cells per ml. These cultures were carried for 14 days. Population curves of the control and "0.01 mg cells" indicated rapid rates of proliferation which closely approximated each other. Contrary to the earlier impressions from short term experiments the 0.01 mg per ml. of acetaldehyde appeared to have no effect, or if any, a slight stimulatory effect. Microscopic observation of the control cells and the "0.01 mg cells" revealed healthy and flourishing cultures.

The "0.05 mg cells", although during the first day or two appeared normal, did not attach readily to the substratum and evidently did not proliferate. By the fourth day the "0.05 mg cultures" were sparse. Cells were detaching and there was inadequate pH adjustment of the medium and on the eighth day the cultures were discarded.

Earle's strain L cells:

After 40 days of exposure to the acetaldehyde treated medium, replicate cultures in Carrel flasks were made of the control and the two groups of experimental cells. The initial inoculum for the cultures was 500,000 cells per ml. These cultures were carried for 30 days. The cells of all three groups appeared healthy throughout the run and the cultures seemingly flourished. For a time, there appeared to be a slight inhibitory effect in the medium containing 0.05 mg per ml, however, the final populations in all three cases were approximately the same. Any harmful or beneficial effects, if existent, were negligible.

HeLa cells:

Replicate cultures were made 45 days after exposure to the acetaldehyde medium. Only the control cells and the "0.01 mg cells" were replicated, for at this time, difficulty was experienced in keeping the "0.05 mg cells" from sloughing extensively. The initial inoculum for both sets of cultures was approximately 350,000 cells. These cultures were maintained for 17 days. Microscopic examinations did not reveal any morphological differences between the two sets of cells. The population curves show comparable proliferation rates and comparable final populations.

Series 2.

Mouse Liver cells:

A second series of replicate cultures of Liver cells were begun following 72 days of exposure. The initial inoculum varied somewhat among these sets. For the control cultures, the inoculum was approximately 600,000 cells per ml; for the "0.01 mg cells", approximately 550,000 cells per ml; and for the "0.05 mg cells", approximately 500,000 cells per ml. Part of the replicated "0.01 mg cultures" and "0.05 mg cultures" in Carrel flasks were returned to the regular control medium at the first medium change following replication.

The "0.01 mg cells" and the "0.01 mg cells which had been returned to normal medium" appeared to proliferate extremely rapidly, and by the sixth day the number of cells had doubled in the former and more than doubled in the latter cultures as compared to the controls. Four days later, however, the cell number had dropped considerably below the number in the control cultures. After this, the increase in population was consistent in both though not quite reaching the maximum of the controls. The possibility appears that with such a rapid rate of proliferation at the beginning, the first maximum population for the "0.01 mg cells" and the "0.01 mg cells which had been returned to normal medium" (hereafter designated as "0.01 N cells") may have been reached. This overcrowding and eventual sloughing may have resulted within the intervening four days before the next count. The final maximum population may in reality have been a second maximum. These cells appeared to be healthy throughout the run.

The population curve for the controls appeared to be typical. These cells remained in fine condition and arrived at their maximum in 13 days.

The "0.05 mg cells" never became established in Carrel flasks hence were gradually lost in subsequent medium changes and had to be discarded. For the most part, they remained rounded and floating. The replicate cultures in T-60 flasks also had to be discarded after 89 days of exposure. The cells would not attach to the flasks. The "0.05 mg cells" which were returned to normal medium (hereafter designated as "0.05 N cells") after three days recovered very rapidly and proliferated at a rate that resulted in a maximum population comparable to the second maximum of the "0.01 mg cells" and "0.01 N cells" at the same time. These cells appeared normal and healthy.

Earle's strain L cells:

Replicate cultures of the second series of L cells were set up after the cells had been exposed for 110 days. The initial inoculum varied among the sets. For the controls, the inoculum was approximately 360,000 cells per ml; for the "0.01 mg cells", nearly 530,000 per ml; for the "0.05 mg cells", nearly 450,000 per ml. As in the second series of Liver cells part of the "0.01 mg cultures" and "0.05 mg cultures" were returned to regular (untreated) medium at the first medium change. The results obtained in this experiment were almost identical to those obtained in the first series. Proliferation rates for all cells were almost the same for the first two weeks. Differences appearing after that were probably of no great significance. All cells appeared to be in good condition. There was no evidence of inhibitory or stimulatory effects of the acetaldehyde.

HeLa cells:

Replicate cultures of HeLa cells were made after the cells had been exposed for 125 days. The inoculum for all the cultures was approximately 275,000 cells per ml. Part of the "0.01 mg cultures" and "0.05 mg cultures" were returned to regular medium at the first medium change.

Proliferation rates for all the cells did not vary greatly during the first seven days. At this time, however, the proliferation rates for the "0.01 mg cells"and "0.05 mg cells" slowed appreciably. This lag in proliferation of the "0.01 mg cells" and the "0.05 N cells" extended over a period of approximately a week; for the "0.05 mg cells" it extended throughout the remainder of the experiment.

Depletion of the cultures of the "0.05 N cells" make it difficult to predict the trend for these cells. There appears the possibility that the "0.01 mg cells" would reach a final population approximating that of the controls. The rates of proliferation for the controls and "0.01 N cells" closely approximated each other and exceeded the rates for the other cells. In the case of the "0.05 mg cells", the acetaldehyde has a definite influence; the rate of proliferation being less and the final population being about one-half that of the controls.

In appearance, the cells at first appeared to be healthy and typically young. As the cultures aged, the cells assumed the typical appearance found in older, crowded cultures. This was true except for the aging "0.05 mg cells" which did not cover the floor of the flask densely and hence were not very crowded; in fact, they appeared rather slender and many had long extensions.